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THE PROOLIGONUCLEOTIDE APPROACH IV : SYNTHESIS OF CHIMERIC PROOLIGONUCLEOTIDES WITH 6 ENZYMOLABILE MASKING GROUPS AND UNEXPECTED DESULFURIZATION SIDE REACTION

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Abstract: Chimeric dodecanucleotides exhibiting a central gap of 6 phosphorothioate internucleoside linkages masked with pivaloyloxymethyl groups and uncharged or charged flanks were synthesized by alkylation of the corresponding phosphorothioate oligos. In total CEM cell extract, they selectively yielded the unmasked charged oligos with a half-time of about 30 hours. Furthermore during alkylation of phosphorothioate linkages a partial desulfurization was observed.

Antisense oligonucleotides and their analogues have been widely used to inhibit gene expression in a variety of systems[1,2]. The two main hurdles for antisense activity are the poor nuclease resistance of natural oligos and their inefficient intracellular uptake. Nuclease resistance problems are almost resolved by the use of modified oligos. It was shown that poor uptake and/or delivery may be mainly related to the polyionic character and the high molecular weight of the oligos. Hence when neutral or reduced charged oligos are used their lipid solubility is increased and thereby their ability to penetrate biological membranes is increased[3]. It is widely believed that a RNase H mediated mechanism contributes at least for a large part to the antisense effect observed in cell cultures[2,4]. However, among modified oligos only phosphorothioate and phosphorodithioate ones are able to elicit RNase H activity. To overcome this limitation, it was suggested to use chimeric methylphosphonodiester/phosphodiester oligos[5].

Previously, we showed that a chimeric methylphosphonate oligo containing a gap of three phosphotriesters with carboxyesterase labile PivaloylOxiMethyl (POM) groups was selectively deprotected in total cell extract with a half-time for complete deprotection of 2.4 hrs[6]. However it was

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observed that the rate of removal of the last POM group was much slower than that of the first one (< 5 min.). This has been tentatively explained by a decrease in affinity of the partially deprotected chimeric oligo for the carboxyesterase enzyme due to the successive appearance of negative charges, since carboxyesterases prefer neutral substrates[7,8].

Since a gap of 5 or 6 phosphodiesters is required to induce mammalian RNase H activity[9], we decided to study the kinetics of deprotection in total CEM cell extract (TCE) of two chimeric oligos (**1** and **2**) bearing the same gap of 6 POM phosphotriesters with either neutral methylphosphonate flanks or with charged phosphodiester flanks. In addition, the oligo **3** was considered[10] as a model, to study the rate of deprotection in TCE of a last enzymo-labile POM group surrounded by negative charges. Furthermore, a prooligo containing a deoxyadenosine residues (TAT trimer **4**) was synthesized in order to evaluate its stability at low pH (*i.e.* pH 1.2 and in gastric juice) against the depurination, for eventual oral delivery of the prooligos.

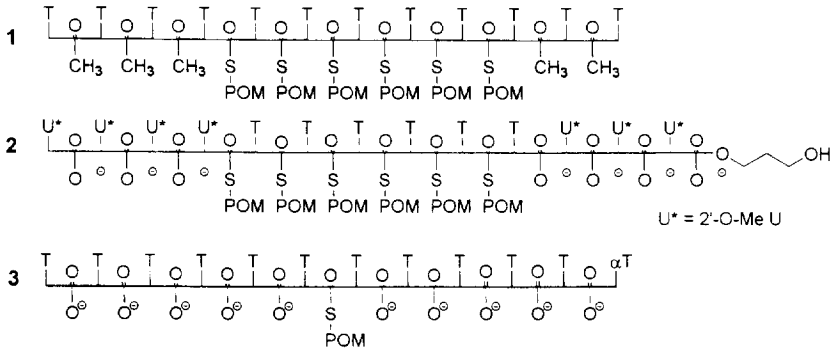

Figure 1 : Schematic structure of prooligo models

As previously described [6,11,12], compounds **1-3** were synthesized through a selective post-oligo synthesis process, using POM iodide as alkylating agent. The reaction was HPLC monitored and the expected alkylated oligos were purified and desalted on Sep Pak C_{18}. Noteworthy, the monitoring and the analysis of the crude reaction mixture was not straightforward due to the presence of chiral phosphorus atoms.

The alkylation was performed in acetonitrile/water with 2,6-lutidine using about one hundred equivalent of POM iodide. A better overall yield of alkylation was obtained for compound **1** (80 % in 4 hrs.) than for **2** (60 % in 6 hrs.).

Hydrolysis of **1**, **2** and **3** in TCE were performed as previously described [6,12] (using on-line cleaning

HPLC method[13]) and half-lives for the first hydrolysis were determined as well as half-times of appearance of the fully unmasked chimeric oligos (**1** and **2**).

Prooligo	HPLC Retention time* (min.)	Half-life in TCE (first hydrolysis)	Half-time of appearance of fully unmasked oligo
1	59.0	84	1860
2	51.0	8	1800
3	31.5	1320	

Table 1 : Half-lives (min.) of prooligo **1** to **3** in total CEM cell extract. * C_{18} Column, 7 min; isocratic of 0.05M TEA Ac pH 7.0, then gradient in 45 min from 0 to 50% acetonitrile in TEA Ac.

As follows from the data presented in table 1, the first hydrolysis is strongly depending on the nature of the substrate but a gap of 6 POM phosphotriesters can be selectively deprotected to the parent phosphorothioate with a $t_{1/2}$ of about 30 hrs whatever the nature of the flanks is. During monitoring by HPLC of the incubation of **1** and **2**, we noticed that each hydrolysis proceeded with its own rate and could be very different from one to another. These data could be explained by the variation of lipophilicity of the substrate compound due to the progressive removal of POM groups. Hence the rate of hydrolysis is the highest when optimal lipophilicity is achieved. Coming now to compound **3**, as a very simple model, HPLC monitoring of the alkylation reaction with POM iodide led us to an unexpected observation. Under the same experimental conditions as for the synthesis of **1** and **2**, about 20 % of the corresponding desulfurized oligo (^{31}P-NMR and coinjection with an authentic sample) was formed along with the expected phosphorothiolate triester (≈ 80 %). However, we clearly observed by HPLC that desulfurization does occur through the formation of the phosphorothiolate triester and not from the phosphorothioate diester. This unexpected finding led us to reconsider carefully the crude alkylation mixture corresponding to **1** and **2** (HPLC analysis) and again formation of some desulfurization products was suspected.

time (h)	**5**	**6a-b**	**7a-b**	**8a-b**	**4**
0.1	98.3	0	1.7	0	0
17	13.7	17.3	11.9	22.4	34.6
30	7.9	13.6	14.3	25.3	38.9
42	0	0	8	27	65

Table 2 : Percentage of different species during alkylation of **5**.

We therefore decided to gain more information on this side reaction by careful HPLC monitoring of the alkylation of a trinucleoside diphosphorothioate (compound **5**, Fig. 3). The synthesis

of **4** was monitored by HPLC/MS coupling, each HPLC peak (Fig. 2 and table 2) was analyzed and identified by MS [14]. Figure 3 shows the reaction scheme; the first alkylation of **5** yielded **6a** and **6b;** a second alkylation yielded **4** and desulfurization products **7a** and **7b,** which were further alkylated to **8a** and **8b** respectively. At this step, we did not observe any desulfurization of **4**. Furthermore, even after extended alkylation time, we did not notice any side alkylation on the adenine residue. Compound **4** was obtained in 65% yield after 42 hours (table 2).

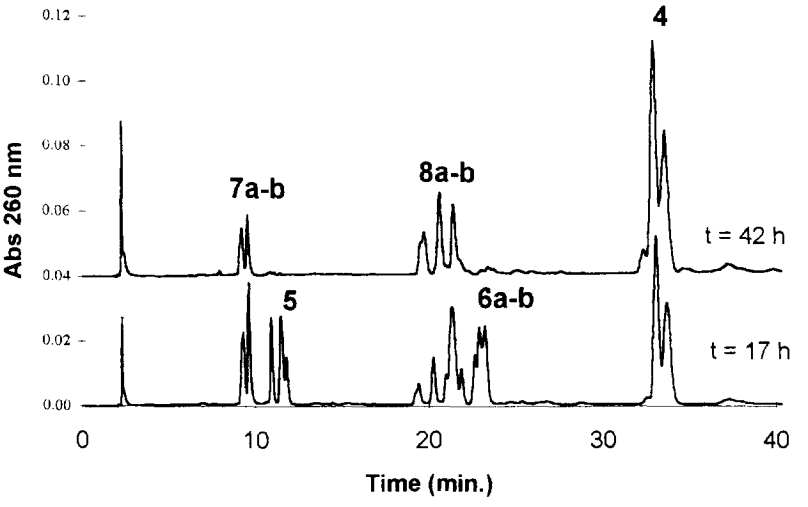

Figure 2: HPLC profiles of the alkylation of TAT trimer **5** by I-POM

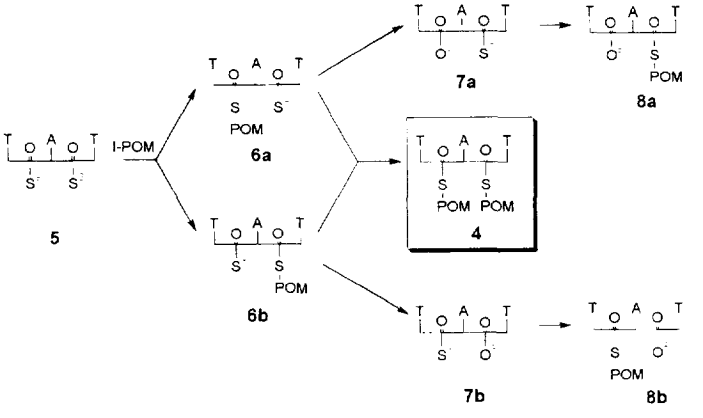

Figure 3 : Schematic representation of alkylation of TAT trimer **5**.

The exact mechanism of this desulfurization has not been firmly established [15], however one can suspect a hydrolysis process associated with concomitant thiolate removal since we have clearly observed by HPLC, on oligo **3** and on other shorter oligos (data not shown) that desulfurization did proceed through the formation of the phosphorothiolate triester. Furthermore we noticed that more desulfurization was always preferentially obtained when chimeric phosphorothioate / phosphodiester oligos are alkylated (versus chimeric phosphorothioate / methylphosphonate oligos).

After isolation of **4**, we studied by HPLC, its behavior in acidic media, in order to evaluate if the introduction of a phosphotriester linkage stabilizes the glycosidic bound.

	Half-life	
	pH 1.2	Gastric juice
4	96 h	70 h
5	3.3 h	ND

Table 3 : Half-life of **4** and **5** in acidic media (pH 1.2 and in gastric juice)

ND: not determined.

Half-lives reported in table 3 show clearly that a strong increase of stability (more than 29 fold) at low pH is reached by the introduction of phosphotriester linkage on the depurination of deoxyadenosine. Such behavior has also been reported for ddA phosphotriesters [16].

In conclusion, we have shown, firstly, that chimeric dodecamers bearing a central gap of 6 enzymolabile POM groups and neutral or charged flanks could be fully unmasked in TCE by carboxyesterase activity with a half-life of about 30 hours. We have to keep in mind that a total CEM cell extract is a rough mimic of a cell content and we can expect that enzyme activities are much more potent in intact cells hence the rate of hydrolysis should be more rapid. Our data suggest that a dodecamer chimeric prooligo bearing a 6 POM gap is compatible with the prooligonucleotide approach.

Secondly, the finding that a desulfurization reaction occurs during alkylation of phosphorothioate oligos is of highest importance for further development of the prooligo approach. It is therefore evident that phosphorothiolate prooligos could not be easily reached through a post-alkylation process. As follows from this communication, further development of the prooligo concept implies that such compounds have to be reached on solid support by a phosphoramidite strategy. Work along this line is in progress in our group.

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14. *Alkylation of **5** was monitored by HPLC MS coupling with negative electrospray mode (m/z) : **4** [M-H]- : 1118, **5** [M-H]- : 889, **6a-b** [M-H]- :1004, **7a-b** [M-H]- : 874, **8a-b** [M-H]- : 988.*
15. *Several controls were done in order to determine the nature of this desulfurization, including addition of EDTA 0.1M to trap possible metal ions or using silanized flasks to avoid any interaction with Si-OH function of glass or addition of sodium iodide to try to increase the amount of desulfurization. All these conditions did not bring any variation. Finally alkylation with other kind of alkylating agents e. g. bromo-phenyl or 4-(iodoacetamido)fluorescein led to fully alkylated oligos without any desulfurization.*
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